REVIEW

Dimerization and oligomerization of G-protein-coupled receptors: debated structures with established and emerging functions

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Abstract

Dimerization or oligomerization of G-protein-coupled receptors (GPCRs) is a novel concept, which may lead to the reevaluation of the actions of pharmacological ligands, hormones, neurotransmitters, and other mediators acting on GPCRs. Although a large number of data obtained using different biophysical, biochemical and structural methods, and functional approaches argue for dimerization or oligomerization of these receptors, several publications criticized the applied methods and challenged the concept. The aim of this paper is to review the data that support the concept of receptor oligomerization, and the most important arguments against it. We conclude that it will require major methodical improvements to obtain decisive proof, whether GPCRs exist in their native membrane environments as

homo- or heterodimeric or oligomeric complexes, in which receptor monomers have stable direct interactions. However, overwhelming amounts of data suggest that many GPCRs exhibit functional properties that require direct or indirect interactions between clustered receptors. Although it is difficult to conclude, about the exact nature of these interactions, dimerization or oligomerization of GPCRs is a useful paradigm for pharmacologists to study properties of receptors, which require functionally important clustering of receptors, such as trafficking of newly synthesized receptors to the cell surface, allosteric modulation of ligand binding, signaling specificity, co-internalization, or cross-inhibition of GPCRs.

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Introduction

The superfamily of the G-protein-coupled receptors (GPCRs) makes up the largest group of membrane receptors (Fredriksson & Schiöth 2005). They respond to a large variety of stimuli, including biogenic amines, peptides, glycoproteins, neurotransmitters, odorants, ions, lipids, nucleotides, light, or even protease enzymes, and they transduce these signals across the plasma membrane by coupling to heterotrimeric G-proteins and G-protein-independent signaling pathways (Bockaert & Pin 1999, Wei *et al.* 2003, Luttrell 2006). Their importance is indicated by the fact that about half of the drugs used in clinical practice directly or indirectly modify the activity of a GPCR (Tyndall & Sandilya 2005, Jacoby *et al.* 2006). The common structural feature of GPCRs is the seven transmembrane (TM) α -helical domains, each composed of 25–35 amino acid residues. The highly hydrophobic helices are connected by three extracellular and three intracellular loops, while the N terminus is extracellular and the C terminus is intracellular. This typical structure gave rise to an alternative name for these molecules, seven TM or 7-TM

receptors, which has the advantage of accommodating the fact that their signal transduction is not limited to G-proteins (Hunyady & Catt 2006, Luttrell 2006).

Based on sequence homology and structural features, GPCRs are traditionally arranged in four groups: family A (or class I) receptors, which includes rhodopsin, activated by small ligands or short peptides; family B (class II) receptors (e.g. secretin-receptor) have a longer N-terminal domain and bind large peptides; family C (class III) GPCRs are characterized by a very large N terminus, and bind small ligands such as glutamate or GABA (metabotropic glutamate receptors, GABA_B-R); and for e.g. the frizzled/smoothed family. A more recent classification divides the receptors into the glutamate, rhodopsin, adhesion, frizzled/taste2, and secretin families (Fredriksson *et al.* 2003). A continually updated list of GPCRs is available at <http://www.iupharbb.org/receptorList/results.php> (Foord *et al.* 2005). The natural ligand is presently unknown for many of these receptors hence they are the so-called ‘orphan’ receptors.

Contrary to a large number of membrane proteins, e.g. tyrosine-kinase receptors, GPCRs were initially considered to

be monomeric entities, coupling to a single G-protein heterotrimer with a 1:1 stoichiometry. In recent years a lot of data accumulated, which challenged this view, suggesting that some GPCRs can exist as dimers or higher-order oligomers (Bouvier 2001, Kroegeer *et al.* 2003, Milligan *et al.* 2003, Terrillon & Bouvier 2004, Milligan & Bouvier 2005, Milligan 2006, Minneman 2007). Most available methods do not distinguish dimerization or oligomerization of GPCRs, therefore we prefer to refer to this process as GPCR oligomerization. While the functional significance of this process is still not completely understood, and in some cases even the applied methodological approaches are debated (Chabre & le Maire 2005, James *et al.* 2006), it is now increasingly accepted that oligomerization of GPCRs can be important for receptor expression and function, including agonist binding, potency, efficacy, and G-protein selectivity. However, whether oligomerization is fundamentally important for all GPCRs is still controversial, which is not surprising, given the large number of receptors belonging to this group and the diverse functions they mediate.

In this paper, our goal is to use examples to present different arguments for and against the concept of receptor dimerization or oligomerization, comparing the advantages and disadvantages of the applied methods, and to integrate some exciting recent data into the picture of this complex and rapidly expanding field.

Indirect indications of GPCR oligomerization

Soon after the direct identification of β -adrenergic receptors by radioligand binding in frog erythrocytes (Mukherjee *et al.* 1975), negative cooperativity was observed among the binding sites, which raised the possibility of physical interactions between individual receptor monomers (Limbird & Lefkowitz 1976). The functional significance of clustered receptors organized by receptor–receptor interactions was first hypothesized a quarter of a century ago (Agnati *et al.* 1982). The activating role for receptor aggregation was also postulated, when an antagonist of the gonadotropin-releasing hormone (GnRH) was converted into an agonist after incubation with a bivalent antibody against the antagonist. The latter results suggested that a pure antagonist can act as an agonist when it is capable of bringing two receptor molecules within a critical distance, between 15 and 150 Å (1.5 and 15 nm; Conn *et al.* 1982). Other observations supporting the idea that GPCRs might form oligomeric complexes came from radiation inactivation, photo-affinity labeling, cross-linking, and gel filtration experiments performed in the 1970s and 1980s (reviewed in Bouvier (2001)).

Another line of evidence came from trans-complementation studies in which the co-expression of two nonfunctional mutant or chimeric receptors resulted in the restoration of function. In a classical study, two chimeric receptors, one containing the first five TM domains of the α_2 -adrenergic receptor and the last two TMs of the M3 muscarinic

acetylcholine receptor (M3-AchR), and the other vice versa, were used (Maggio *et al.* 1993a). When expressed alone, both of these receptors were nonfunctional, showing no binding of muscarinic or adrenergic antagonists. Co-expression of the two constructs resulted in a partial restoration of binding for both of the receptors, and signaling through the M3-AchR. In the same study, a similar restoration of function was observed between two differently mutated M3-AchRs as well, suggesting both hetero- and homo-oligomerization of this receptor. In a similar experiment, two mutants of the type 1 angiotensin II receptor (AT₁R) were studied (Monnot *et al.* 1996). Both K102A (in TM3) and K199A (in TM5) point mutant receptors did not bind the peptide agonist or nonpeptide antagonist of the AT₁R when expressed alone, while co-expression of the two restored the binding site, but not the G-protein activation. These papers led to the idea of the ‘domain-swapping’ model of dimerization in which two independent folding units of the receptor separate and recombine between the two protomers of the dimer (Fig. 1B). In the ‘contact dimerization’ or ‘lateral packing’ model (Fig. 1A), the integrity of the receptor monomers is maintained (Kroegeer *et al.* 2003).

In the case of domain-swapping, the folding units are believed to be made up from the first five and the last two TM domains, connected by the third intracellular loop as a ‘hinge’, allowing the units to separate (Gouldson *et al.* 2000). This is supported by the observation that truncated M2- and M3-AchRs, split in their third intracellular loop into N- and C-terminal fragments, which do not bind ligands when expressed alone, can reassemble into functional receptors capable of binding and signaling (Maggio *et al.* 1993b). Alternatively, the second intracellular loop can also act as a ‘hinge’, as indicated by studies involving M3-AchR (Schoneberg *et al.* 1995) and AT₁R (Monnot *et al.* 1996).

Other studies did not find evidence of such functional rescue between co-expressed V2 vasopressin (Schulz *et al.* 2000) or D₂ dopamine receptor mutants (Lee *et al.* 2000), despite demonstrating dimerization of the molecules. These findings support the concept of contact dimerization in which the GPCRs directly contact each other using interaction sites on the exterior of the TM domains. It is quite possible that the two kinds of dimers may co-exist although the contact dimers would be more favorable energetically, as their formation does not require the rearrangement of the TM domains between the receptors. This notion is supported by the observation that after a highly conserved extracellular disulfide bond was mutationally disturbed in the V2 receptor, it was able to form a domain-swapped construct with a co-expressed receptor fragment (Schulz *et al.* 2000). Quite often, the number of binding sites recovered in domain-swapping experiments is much lower than that of the wild-type receptor, which also suggests the energetically unfavorable nature of this kind of interaction (Bakker *et al.* 2004).

Theoretically, both models of dimerization may be extended to the formation of higher-order oligomeric structures. In the case of contact dimers, a second set of

Figure 1 Possible mechanisms of the formation of GPCR dimers and oligomers. (A) In the contact dimer or lateral packing model, GPCRs directly contact each other via exterior residues of the transmembrane domains. These dimers may associate at other interacting surfaces leading to the formation of receptor oligomers. (B) The domain-swapping model requires changes in the integrity of the monomers by swapping of independently folded transmembrane domains between GPCR monomers. Theoretically, lateral packing dimerization of domain-swapped dimers would also make possible the formation of higher-order oligomers.

interaction points would provide the link between the dimers, whereas domain-swapped dimers could form oligomers by lateral packing (Fig. 1).

Biochemical methods to detect GPCR oligomerization

Co-immunoprecipitation is the most frequently used biochemical approach to detect the oligomerization of GPCRs. This is commonly accomplished using differentially epitope-tagged molecules expressed in recombinant systems. In the first application of this technique to study receptor

dimerization, β_2 -adrenergic receptors (β_2 AR) tagged with influenza hemagglutinin (HA)- and myc-tags were co-expressed in Sf9 cells, and after immunoprecipitation with an anti-myc antibody, the dimer could be detected using an anti-HA antibody (Hebert *et al.* 1996). To control the selectivity of the interaction, a myc-tagged M2 muscarinic Ach receptor was also co-expressed with HA- β_2 AR, and these two could not be co-immunoprecipitated.

With this technique, the cells expressing the two receptors are solubilized and the lysate is incubated with an antibody directed against one of the receptors, or the epitope tag fused to the receptor. The complex is bound to an appropriate medium, electrophoresed, blotted, and visualized using an antibody

against the other receptor of interest, or its appropriate tag, displaying the GPCRs which form oligomers. The whole process is usually performed in reverse as well, immunoprecipitating with the second antibody and immunoblotting with the first, in order to confirm the specificity and reciprocity of the interaction. However, in many cases, glycosylation of GPCRs prohibits the estimation of the molecular weight of the receptor, and the interacting GPCRs are detected as higher molecular weight complexes. In such cases, the observed interaction may be due to the *in vivo* formation of larger molecular complexes, therefore in many cases co-immunoprecipitation is not appropriate to draw a conclusion about direct interactions between receptor molecules.

There are a great number of studies documenting the homo- and hetero-oligomerization of different GPCRs using this or similar methods (reviewed in Kroeger *et al.* (2003)). Some studies suggested a marked specificity of the interaction, e.g. κ -opioid receptors (OR) dimerized with the δ -OR, but not with the μ -OR (Jordan & Devi 1999), others showed a promiscuous dimerization, such as the 5HT_{1A} serotonin receptor, which dimerized with distantly related GPCRs (Salim *et al.* 2002), and there were negative findings as well, for example the *N*-formyl peptide receptor was shown not to form homodimers (Gripentrog *et al.* 2003). Immunoprecipitation can be used to detect oligomeric GPCR complexes, as shown by a study on M2-AchR, where three differently epitope-tagged forms of the receptor (using FLAG[DYDDDDDK]-, HA-, and myc-tags) were co-expressed in, and co-immunoprecipitated from Sf9 cells (Park & Wells 2004). This paper suggested the existence of trimeric or higher order complexes, co-existing with monomeric receptors, which were also observed.

While co-immunoprecipitation is a commonly used method for the detection of GPCR oligomers, it does have several drawbacks, mainly relating to the lysis and solubilization steps. First, it requires the solubilization of the cells; therefore, it obviously cannot be used to study interactions in living cells. Second, the highly hydrophobic nature of the seven TM domains makes solubilization using detergents a difficult task and GPCRs can form aggregates during this step (Ramsay *et al.* 2002, Salim *et al.* 2002). One approach to control this phenomenon is to mix the membranes prepared from cells individually expressing one receptor or the other, and solubilize them, in which case no co-immunoprecipitation should be observed (Jordan & Devi 1999). However, the problem may persist if the aggregation occurs during the solubilization process due to inappropriate detergent concentration (Chabre & le Maire 2005). On the other hand, using too much detergent may disrupt the existing interactions between receptors. Another possibility is to use a membrane-impermeable cross-linking agent before the solubilization, as this can stabilize preformed oligomers and demonstrate that they existed before the lysis (Hebert *et al.* 1996, Cvejic & Devi 1997). However, the production of free sulfhydryl groups during the lysis can also lead to artificial associations independent of receptor oligomerization. This can especially happen when reducing agents such as

dithiothreitol or 2-mercaptoethanol are present in the solubilization buffer, therefore capping agents such as iodoacetamide are often used to prevent this. The third problem is also related to the use of detergents, as incomplete solubilization can result in small remaining membrane patches in the supernatant, which may contain both co-expressed GPCRs that are not actually physically in contact. To prevent this, it is necessary to ensure the production of a fully soluble fraction, which can be achieved by thorough centrifugation after detergent extraction, for at least 60 min and over 100 000 *g* (Gines *et al.* 2000). Quite often, the centrifugation is only done in a microcentrifuge producing around 14 000 *g* and for a shorter time (Salim *et al.* 2002). Lacking the necessary equipment for this step, it has been suggested that passage of the lysate through a 0.22 μ m filter may be sufficient to remove the remaining membrane fragments (Milligan & Bouvier 2005). An additional problem is caused by the glycosylation of some GPCRs, which can produce artificial aggregation of the receptors during the boiling step used to disrupt the immunoglobulin complexes in immunoprecipitation studies. This problem can be reduced by disrupting the complexes at lower temperatures (Smith *et al.* 1998).

Another major problem arises from the high levels of overexpression often used in these types of experiments. Such artificial levels may induce interactions which would not happen in a physiological environment. This can potentially be avoided by using cell lines stably expressing the studied receptors at a low level, or by changing the strong promoter of the expression vector to a weaker one (e.g. herpes simplex virus thymidine kinase promoter, providing a low-level constitutive expression), or by using an inducible expression system. Perhaps the physiologically most relevant information could be gained from cell lines or tissues endogenously expressing the studied GPCRs. Indeed, one of the greatest advantages of co-immunoprecipitation is that it can be used to directly detect receptor oligomers in *ex vivo* tissue samples, as it was demonstrated for example in the case of the GABA_{B1} and GABA_{B2} receptors (Kaupmann *et al.* 1998). However, a major obstacle to study GPCR oligomerization in endogenously expressing cells can be attributed to the paucity of quality antibodies against receptors. Specificities of antibodies against some GPCRs are debated (Smith *et al.* 1998, Thomas 1999, Fredholm *et al.* 2007). As mentioned in a recent review, it is very difficult to raise useful antibodies to GPCRs, which may be a consequence of the short length and constrained conformation of the extracellular loops, and the main problem with them is the high incidence of false positivity (Fredholm *et al.* 2007). Consequently, it is possible that some findings regarding receptor localization or oligomerization in native tissues may eventually turn out to be artifacts caused by nonspecific interactions of these antibodies.

Although co-immunoprecipitation may serve as a starting point to analyze oligomerization of GPCRs in native tissues, considering the methodical difficulties, such as selection of the appropriate antibody, the detergent and its concentration, additional methods should be used to verify the detected interaction.

Resonance energy transfer (RET)

The development of techniques based on RET, and their application to the investigation of interactions between GPCRs, revolutionized the field of receptor oligomerization. These methods are able to sensitively detect protein–protein interactions in live cells, and in real-time, allowing monitoring of the kinetic and dynamic properties of GPCR complexes (Pfleger & Eidne 2005).

RET is the nonradiative transfer of energy from a donor molecule to an acceptor molecule as a result of electromagnetic dipole–dipole coupling (Förster 1948). In the case of fluorescence RET (FRET), the energy donor is a fluorescent molecule, excited by exposure to light of a characteristic wavelength, transferring the emitted energy to a fluorescent acceptor molecule (Fig. 2A). The donor molecule can also be an enzyme, Renilla luciferase (Rluc), which causes energy to be released upon oxidation of its substrate, coelenterazine to coelenteramide. The resulting energy transfer to a fluorescent acceptor molecule is called bioluminescence RET (BRET; Fig. 2B).

There are several prerequisites for RET: first, the emission spectrum of the donor molecule should significantly overlap with the excitation spectrum of the acceptor molecule; second, both the donor and the acceptor should be properly oriented relative to each other; and third, they should be in close molecular proximity, effectively <100 Å. As the efficiency of the energy transfer is inversely proportional to the 6th power of distance between the dipoles, this method is especially suited to detect real interactions between proteins, opposed to simple co-localization studies, which are limited by the low spatial resolution of light microscopy. However, the dependence of RET on the proper orientation of the dipoles means that the donor and the acceptor molecules must have a significant degree of freedom in their movement, to make sure that their relative orientation allows the development of RET in at least part of the time. In the case of GPCR fusion proteins, this freedom of movement can be restricted which may impair the RET efficiency. This means that two tagged proteins may fail to produce a RET signal even if they interact with each other. To avoid this problem, it may be necessary to design fusion proteins with different linker sequences (length and composition) and test them for RET.

FRET and BRET can be measured on cell populations by scanning spectroscopy or an appropriate microplate reader or on single cells by microscopy. Microscopy is often used to detect FRET, while BRET is usually measured in plate readers. A great number of GPCRs has been reported to homo- or hetero-oligomerize using these techniques (reviewed in Pfleger & Eidne (2005)).

FRET

The green fluorescent protein (GFP) isolated from the jellyfish *Aequorea victoria*, and its variants are important tools

in cell biology, allowing the monitoring of different tagged proteins in living cells (Chudakov *et al.* 2005). The altered spectral properties of these proteins allow the selection of FRET pairs, of which the most widely used is the cyan fluorescent protein (CFP) as donor and the yellow fluorescent protein (YFP) as acceptor. In this case, CFP can be excited with blue light (around 430 nm), and YFP emission can be observed in the yellow range (around 530 nm; Fig. 2A). This was the combination used in the first application of the FRET method to study GPCR oligomerization – in this case, the fusion proteins were constructed as C-terminal fusions to a truncated form of the yeast α -factor receptor, lacking their cytoplasmic C-terminal regulatory domains (Overton & Blumer 2000). Efficient FRET between the receptors due to stable association rather than a collisional interaction was demonstrated in intact cells and membranes. The equilibrium between monomers and oligomers was unaffected by binding of agonist, antagonist, or G-protein heterotrimers. When the tags were fused to the full-length receptors, no FRET signal was detected, which indicated the dependence of FRET on the interfluorophore distance, orientation or mobility.

While the CFP/YFP donor/acceptor pair is the most widely used combination for the detection of FRET between GPCRs, other fusion protein pairs can also be used for this purpose, for example: CFP/GFP (Stanasila *et al.* 2003), GFP/DsRed (Dinger *et al.* 2003), GFP/YFP (Canals *et al.* 2003), and YFP/red fluorescent protein (RFP; Latif *et al.* 2001). Alternatively, fluorophores (e.g. fluorescein/rhodamine) conjugated to antibodies can also be used. The antibodies can be directed against epitope tags incorporated into the receptor (Rocheville *et al.* 2000), or a receptor-specific primary antibody can be used together with a fluorophore-conjugated secondary antibody (Patel *et al.* 2002a). This method allows endogenously expressed GPCRs to be studied, which can be an advantage of FRET over BRET, but it depends on the availability of a suitably specific antibody against the receptor.

There are several factors that complicate the interpretation of FRET studies. First, the light used to activate the donor molecule may directly excite the acceptor as well. Second, the donor molecule's emission may leak through into the channel used to detect the emission of the acceptor. Third, the FRET efficiency is affected by the expression levels of the donor and the acceptor molecules, while autofluorescence and photobleaching can further complicate the measurements. To overcome the above problems, appropriate correction factors should be used, which can be calculated from separate measurements of the fluorophores, as it was described e.g. in a study regarding the homo-oligomerization of the C_{5a} complement factor receptor (Floyd *et al.* 2003).

FRET can be measured on populations of cells (either in suspension or adherent), but a major advantage of this technique is that it can be combined with microscopic imaging, using both widefield and confocal microscopes. This allows the study of subcellular compartments, and makes it possible to limit the data acquisition e.g. to the plasma

Figure 2 Illustration of the principle of resonance energy transfer methods. (A) In the case of FRET, a donor fluorophore (in this case CFP) is excited with an external light source, resulting in the emission of blue light, if there is no acceptor molecule nearby (upper panel). If an appropriately oriented acceptor molecule (in this case YFP) is in close proximity (<100 Å), FRET occurs resulting in the emission of a yellow-shifted light (lower panel). (B) In the case of BRET, the donor molecule is the Rluc enzyme, which, upon oxidation of coelenterazine, emits a blue light (upper panel). If the donor is in molecular proximity with an acceptor molecule (in this case YFP) resonance energy transfer occurs, which leads to emission of the light characteristic of the acceptor molecule (lower panel).

membrane. This can be particularly advantageous in the case of GPCR oligomerization, if one would like to separate the oligomerization that occurs during biosynthesis (in the endoplasmic reticulum (ER) and the Golgi) from that

on the cell surface (Herrick-Davis *et al.* 2004). The direct confocal measurement of FRET on live cells also needs correction factors to be used. A method has been developed for this correction, which uses online corrective

measurements to optimize the detection of FRET in the case of the CFP/YFP pair (van Rheenen *et al.* 2004). Imaging can also be combined with various photobleaching approaches, discussed below, which increase the sensitivity of FRET detection.

However, not all FRET studies regarding GPCR oligomerization support the existence of oligomers. In a recent paper, a modified version of FRET microscopy was used to study the distribution of neurokinin-1 receptor (NK1R) on the plasma membrane (Meyer *et al.* 2006). This technique, called acyl carrier protein labeling, allows the post-translational attachment of different fluorophores to the studied protein in living cells, the ability to selectively label receptors on the cell surface, and control of the donor–acceptor ratio. Combining this method with single-cell FRET measurements, the authors excluded the presence of constitutive or ligand-induced homodimers or oligomers of NK1Rs, and revealed that the monomeric receptors concentrated in microdomains, which constituted about 1% of the cell membrane and were sensitive to cholesterol depletion.

While most of the RET-based techniques are generally unable to determine whether the quaternary structure is restricted to dimers, or higher-order oligomers also exist (Milligan & Bouvier 2005), there are advances in this field which help to overcome this problem. The FRET methodology can be extended to follow the energy transfer through three different molecules in a sequential manner (e.g. CFP/YFP/RFP), which is called three-chromophore FRET or 3-FRET (Galperin *et al.* 2004). Using this method, it was recently shown that the α_{1B} -adrenergic receptor is able to form oligomeric rather than only simple dimeric complexes in living cells (Lopez-Gimenez *et al.* 2007).

Photobleaching FRET (pbFRET)

Photobleaching is an intrinsic property of a fluorophore, characterized by the fading of the fluorescence, which is caused by an irreversible photochemical destruction of the molecule upon continuous exposure to the excitation light. While this phenomenon usually hampers fluorescent measurements, it can be used for the detection of RET, as the rate of photobleaching is comparable to that of FRET (i.e. on the nanosecond time scale), and it always occurs from the excited state of the fluorophore; therefore, this process effectively competes with RET. Either donor or acceptor pbFRET can be measured and these techniques allow the direct demonstration of FRET without using the numerous correction factors needed for conventional FRET studies.

In the case of donor pbFRET, the donor fluorophore is photobleached by an intense illumination, and its fluorescence is measured in the absence and presence of the acceptor. If the two molecules are close enough for RET to occur, it competes with the photobleaching process, which can be measured as a decrease in the rate of photobleaching, shown by an increase in the photobleaching time constant.

This technique has been used to demonstrate the homodimerization of the human somatostatin receptor (SSTR) subtype 5 (SSTR5; Rocheville *et al.* 2000). N-terminally HA-tagged SSTR5s were expressed in CHO cells, and pbFRET was measured between fluorescein- and rhodamine-conjugated anti-HA antibodies. In cells expressing high levels of SSTR5 on the membrane ($B_{\max} = 800 \pm 90$ fmol/mg protein), significant constitutive dimerization was observed in the absence of agonist, and it could be further stimulated with agonist treatment. In the case of a cell line expressing a fivefold lower concentration of receptors ($B_{\max} = 160 \pm 30$ fmol/mg protein), the cells displayed insignificant effective FRET efficiencies in the basal state, suggesting that monomers predominate in the absence of agonist when the receptor is expressed at levels in the range of endogenous SSTR concentrations. Agonist treatment resulted in a dose-dependent increase in FRET efficiencies, suggesting that oligomerization, or conformational rearrangement of preformed oligomers, is induced by the binding of the agonist. This observation reiterates the importance of physiological receptor expression levels in oligomerization studies (Patel *et al.* 2002b).

In the case of acceptor pbFRET, donor and acceptor emissions are measured before and after the photobleaching of the acceptor. If the fluorescence of the donor increases after the destruction of the acceptor, this is taken as evidence of FRET occurring between the donor and acceptor fluorophores. This method has been used to demonstrate agonist-dependent microaggregation of the GnRH receptors (GnRHR), C-terminally tagged either with GFP or RFP, both in fixed and in live cells (Cornea *et al.* 2001). Another study used FRET between C-terminal CFP and YFP tags, in addition to co-immunoprecipitation, to show heterodimerization between endothelin A and endothelin B receptors expressed in HEK293 cells (Gregan *et al.* 2004).

A possible obstacle to the latter type of experiments (i.e. acceptor pbFRET using a CFP/YFP pair) was revealed in a paper in which the photoconversion of YFP into a CFP-like species was observed upon photobleaching of YFP (Valentin *et al.* 2005). According to this study, during pbFRET experiments, any increase in the CFP channel could arise from either FRET or from YFP to CFP photoconversion. To further complicate the picture, other groups failed to reproduce this phenomenon, and dismissed the idea of YFP photoconversion (Thaler *et al.* 2006), but a recent paper again confirmed the production of a CFP-like species, and concluded that until more is understood about the nature of this fluorescent by-product, careful controls need to be done for acceptor pbFRET studies involving YFP (Kirber *et al.* 2007).

(Homogeneous) time-resolved FRET ((h)trFRET)

Time-resolved FRET is another method designed to avoid some of the problems associated with the measurement of direct FRET intensity. This approach takes advantage of the

long-lived fluorescence characteristics displayed by certain lanthanide compounds such as terbium or europium. The prolonged fluorescence of these compounds allows the excitation and the detection of emission to be temporally separated, during which time the signal arising from autofluorescence and direct excitation of the acceptor dies down. This technique uses antibodies labeled with europium (Eu^{3+}) chelate (McVey *et al.* 2001) or cryptate (Maurel *et al.* 2004) as donors, and allophycocyanin- or Alexa Fluor 647-labeled antibodies as acceptors. The use of antibodies ensures that only the receptors on the cell surface are detected, but it also serves as a limitation, since, as described above, development of antibodies against GPCRs is often difficult, and specific antibodies are not available for many of these receptors. However, the antibodies can be directed against epitope tags incorporated into the receptor constructs, or receptor-specific primary antibodies can be used in conjunction with labeled secondary antibodies. The Eu^{3+} label is excited at 320 nm, and the emissions are monitored after a 50 μs delay for 200–400 μs at 615 nm for Eu^{3+} emission and at 665 nm for RET. As the donor dipole is effectively rotating in the chelate or cryptate, the emission is unpolarized, which greatly reduces the orientation dependence of FRET, making distance determination more accurate.

While the trFRET measurements usually include washing steps to remove unbound antibodies, e.g. in the study demonstrating δ -OR homo-oligomers at the plasma membrane (McVey *et al.* 2001), homogenous assays without washing steps are also possible, thanks to the high sensitivity of this method and to the low concentration of antibodies required (Maurel *et al.* 2004). This htrFRET approach is particularly suited to high-throughput screening protocols.

Fluorescence lifetime imaging (FLIM)

The fluorescence lifetime of a fluorophore is defined as the average time that a molecule remains in an excited state before returning to the ground state. This is a property of the individual fluorophore; it is unaffected by the change in probe concentration or excitation intensity but is influenced by changes in the cellular environment, such as changes in pH and ion concentration, and is also affected by FRET. In a FRET-FLIM approach, the occurrence of FRET is measured by monitoring the change in donor lifetime in the presence and absence of acceptor, and if FRET occurs, the donor lifetime is shortened. While it requires the use of specialized equipment, FRET-FLIM has some significant advantages over intensity-based FRET approaches (Wallrabe & Periasamy 2005), and is particularly suited for *in vivo* studies because it requires a low excitation intensity (avoiding photobleaching) and low levels of fluorescent proteins can be detected. The two major instrumental methods for measuring fluorescence lifetimes are the frequency domain and the time domain measurements. Either method can be used in one-photon or two-photon FRET-FLIM microscopy, the latter being more suited for the imaging of live cells thicker specimens.

The FRET values from GFP fluorescence decay can be calculated with spatial resolution, which enables the proportion of interacting partners to be determined, as it was demonstrated in the study of homo- and hetero-oligomerization of β -arrestin molecules in living cells (Storez *et al.* 2005).

FRET between ligands

Another possible method to study GPCR oligomerization involves the use of ligands conjugated to fluorophores. In a paper investigating the self-association of luteinizing hormone (LH) receptors, FRET was measured between LH or human chorionic gonadotropin (hCG) molecules labeled with fluorescein and rhodamine (Roess *et al.* 2000). The observed FRET was higher between LH receptors binding the fluorescent hCG molecules, than between the ones binding LH molecules. In another study, fluorescein- and Texas Red-conjugated somatostatin molecules were used to show that SSTR homo- and hetero-oligomers must be occupied by the two fluorescent ligand molecules, and the ligands must reside within 100 Å of each other (Patel *et al.* 2002a). This technique can be potentially used to study interactions between endogenous receptors, particularly regarding the recent advances in the various available fluorophores (Giepmans *et al.* 2006).

BRET

BRET is a naturally occurring phenomenon in several marine animals, such as the jellyfish *A. victoria* and the sea pansy *Renilla reniformis*. These organisms produce a substrate, coelenterazine, which is enzymatically oxidized into coelenteramide, with the subsequent release of bioluminescent light. In *A. victoria*, the reaction is catalyzed by the aequorin enzyme, resulting in a peak emission at 486 nm, while in *R. reniformis* the Rluc enzyme produces light with an emission maximum at 480 nm. In both organisms, the energy is transferred to the appropriate GFP molecule (*Aequorea* or *Renilla*), which results in the emission of green light (emission maximum at 509 nm) instead of the original blue produced in the enzymatic reaction (Hastings 1996).

The first application of BRET to the study of protein interaction used Rluc as energy donor, and enhanced YFP (eYFP) as acceptor to demonstrate the dimerization of a circadian clock protein, KaiB, expressed in *E. coli* (Xu *et al.* 1999). In this configuration, the peak emission of eYFP can be detected at ~530 nm, and the Rluc at 480 nm. The method was quickly applied to the investigation of GPCRs, and the data suggested a constitutive homodimerization of human β_2 -adrenergic receptors ($\beta_2\text{AR}$) expressed in HEK293 cells (Angers *et al.* 2000), in the same year when FRET was first used to study GPCR dimerization (Overton & Blumer 2000). BRET soon became a widely used method to monitor the interactions of GPCRs (Pfleger & Eidne 2003), and the (homo- and hetero-)dimerization of various receptors were reported (reviewed in Pfleger & Eidne (2005)).

In the classical BRET assay (BRET¹), the substrate is coelenterazine h (this is a synthetic coelenterazine analogue with increased luminescence intensity compared with the native molecule) and the acceptor is eYFP (Fig. 2B). In this configuration, Rluc and eYFP emit light between 475–480 nm and 525–530 nm respectively resulting in a relatively poor spectral resolution (difference between donor and acceptor emission maxima) of 45–55 nm. Also, as Rluc produces a broad emission peak that substantially overlaps the YFP emission, the signal to background ratio of the system is suboptimal. In an effort to overcome these problems, the BRET² methodology (Perkin-Elmer, Waltham, MA, USA) uses a coelenterazine derivative called DeepBlueC as substrate, and GFP² (a modified form of GFP) as acceptor. In this case, the donor emission is at 395 nm and the acceptor emission is at 510 nm resulting in a better spectral resolution (115 nm) and an improved signal-to-noise ratio (Ramsay *et al.* 2002). However, it should be noted that the absolute signals produced are much lower in this configuration than in BRET¹, which decreases the detection sensitivity, and they are decaying at a very fast rate, which makes BRET² impractical for kinetic measurements. It is possible to monitor the two kinds of BRET simultaneously in the same cells (Perroy *et al.* 2004), which may be adapted to study the stoichiometry of GPCR oligomerization. For long-term kinetic studies, a new ‘extended’ BRET (eBRET) methodology may be used (Pfleger *et al.* 2006). This technique uses a protected form of coelenterazine h, termed EnduRen (Promega), which is metabolized to coelenterazine h by esterases within cells, allowing the monitoring of protein–protein interactions for several hours in cells kept at 37 °C, in the presence of serum (which greatly increases the autoluminescence of conventional coelenterazine h and DeepBlueC).

BRET has several advantages and some disadvantages when compared with FRET (Boute *et al.* 2002). Most of the advantages come from the lack of an excitation light source: excitation light can damage live cells, induce autofluorescence, and cause photobleaching of the fluorophores. Moreover, the analysis of FRET signal is complicated by the direct excitation of the acceptor molecule, resulting in a higher background signal. As this background can be eliminated in BRET, this method is theoretically more sensitive than FRET, which was demonstrated by a noncompetitive homogenous bioluminescent immunoassay that was ten times more sensitive than a comparable FRET assay (Arai *et al.* 2001). The disadvantages include the fact that since BRET is always measured between fusion proteins, it can only be performed in recombinant systems. Probably the biggest limitation of this technique is that the low amount of light emitted in the reaction is below the sensitivity of most of the presently available imaging equipment, and thus it cannot easily provide information about the subcellular localization of the observed protein–protein interactions. This can be especially interesting in the case of GPCR oligomerization, as oligomerization can happen early in the biosynthetic pathway, on the plasma membrane, or in endocytic vesicles. The obligate use of expressed proteins can further aggravate

this problem, because overexpression could conceivably induce nonspecific interactions in the ER and the Golgi, or between misfolded proteins targeted to degradation. With recent advances in the field of imaging instruments, this is now beginning to change, as now not only single-cell BRET signals can be detected, as it was demonstrated for β_2 AR, and melatonin MT1 and MT2 homodimers (Ayoub *et al.* 2002), but even subcellular BRET imaging was shown using the agonist-dependent interaction between the V2 vasopressin receptor and β -arrestin 2 as a proof of principle (Coulon *et al.* 2008).

As the movement of membrane proteins is restricted to two dimensions, opposed to cytosolic proteins, it is possible that two expressed GPCRs display an aspecific BRET signal due to ‘molecular crowding’ in the membrane. This problem is especially worrying in the case of heterologous systems, where the expression levels can be very high, and the ER and Golgi may contain large amounts of proteins at various stages of maturation, possibly contributing significantly to the detected BRET signal (Terrillon *et al.* 2003). This necessitates the use of appropriate negative controls. Another question is the relative affinity of the interacting partners, which cannot be determined from BRET experiments employing a single concentration of Rluc- and YFP-tagged GPCRs, because the intensity of a BRET signal depends on both the distance and the orientation of the dipoles. An answer to these questions was provided in the form of quantitative BRET experiments (Mercier *et al.* 2002). In these kinds of studies, the cells are transfected with different concentrations of donor- and acceptor-linked receptors (usually a fix amount of donor and increasing amounts of acceptor), and the BRET signal is plotted as a function of the acceptor/donor ratios, optimally calibrated to cell surface receptor expression derived from radioligand binding. In the case of a specific interaction, the detectable BRET should increase with the increasing acceptor/donor ratios until a saturation level is reached, representing the state when every donor molecule is interacting with an acceptor. The hyperbolic ‘saturation BRET’ curves that can be plotted from such an experiment resemble those of classical saturation ligand-binding studies. The acceptor/donor ratio resulting in half maximal BRET signal (BRET₅₀) is characteristic of the affinities of the interacting partners, and thus can be used to compare the propensity of the studied GPCRs to form oligomers (Ayoub *et al.* 2004). In the case of nonspecific interaction, the so-called ‘bystander’ BRET signal displays a quasi-linear relationship with the increasing acceptor/donor ratio, which may saturate at a very high ratio. These experiments showed that oligomerization can happen at physiologically relevant expression levels. The data from saturation curves can also be used to gain information about the oligomerization states of the receptors (Mercier *et al.* 2002), using models of energy transfer quenching (Veatch & Stryer 1977). The specificity of a given interaction can also be studied using BRET competition experiments in which the BRET signal detected between two interacting proteins at a given acceptor/donor

ratio can be diminished by the co-expression of untagged partners, but not with a noninteracting protein (Ayoub *et al.* 2002). A third way to differentiate between specific and nonspecific interactions is to vary the expression levels of the tagged receptors at a fixed acceptor/donor ratio, in which case the specific partners display a constant BRET signal over a wide expression range, while the nonspecific interaction increases with the levels of the receptors (Mercier *et al.* 2002).

Recently, a 'rigorous experimental framework' has been proposed for BRET experiments studying GPCR oligomerization, which, among others, suggested that a lot of previously reported interaction was actually nonspecific and that saturation BRET experiments should be done in a way that keeps the total receptor number constant (James *et al.* 2006). A conclusion of the paper was that the studied family A GPCRs exemplified monomeric behavior. This study raised a considerable debate (Bouvier *et al.* 2007) and experimental challenge (Salahpour & Masri 2007). While the need for proper controls and careful experimental design cannot be overestimated, BRET experiments remain a very powerful tool for the detection of GPCR oligomerization (Marullo & Bouvier 2007). However, we have to keep in mind that although homo- and heteromeric interactions were detected between a number of different GPCRs using RET methods, these methods actually detect molecular proximity (and orientation). Therefore, although a properly controlled RET signal indicates molecular proximity between the studied molecules, based on these experiments it is difficult to conclude about its structural bases. RET signals may reflect the presence of dimers or oligomers, but it is very difficult to definitely rule out, using these methods, the clustering of receptors in membrane microdomains, where molecular complexes are organized by interacting molecules without the direct interaction between receptor molecules.

Bimolecular fluorescence complementation (BiFC)

BiFC is an alternative approach to the detection of protein–protein interactions in living cells (Kerppola 2006). It is based on the reconstitution of a fluorescent protein molecule upon reassociation of its two nonfluorescent fragments (Hu *et al.* 2002). For example, if YFP is split into N-terminal (172 amino acid residue) and C-terminal (67 amino acid residue) fragments, neither of these molecules displays fluorescent properties when expressed alone. Co-expression of the fragments linked to interacting proteins allows the partial reformation of YFP with the concomitant appearance of the fluorescent signal. The main advantage of this method is that the interaction can be detected with a simple fluorescence microscope and therefore a specialized imaging equipment is not required. Also, the orientation requirements of the fragments are less stringent when compared with FRET. A limitation of the method is the time required for the fragments to form a functional protein, which means that this method is not really suitable to detect fast changes in the

interaction between proteins. This method was used to detect homodimers of the α_{1B} -adrenergic receptor (Lopez-Gimenez *et al.* 2007). An extension of this technique, called multicolor fluorescence complementation, uses fragments from different fluorescent molecules which form complexes with distinct spectral properties, allowing the simultaneous visualization of multiple protein interactions (Hu & Kerppola 2003).

GPCRs often interact with different accessory proteins that modulate their function (Bockaert *et al.* 2004, Parameswaran & Spielman 2006). A combination of BiFC with BRET was recently described to analyze the stoichiometry of such complexes in the case of the calcitonin gene-related peptide receptor (Heroux *et al.* 2007), which requires the association with the single TM receptor activity-modifying protein-1 (RAMP1) to reach the cell surface and be active. The authors used these techniques to elegantly demonstrate that the functional receptor at the plasma membrane consists of at least two molecules of the GPCR and one RAMP1 molecule. This approach can also be used to study the existence of higher-order GPCR complexes.

Structural studies

The most tangible evidence for GPCR dimerization came from atomic force microscopy (AFM) studies of membranes from murine rod outer segment (ROS) disks, which demonstrated complex *in situ* quaternary organization of rhodopsin molecules (Fotiadis *et al.* 2003a, Liang *et al.* 2003). The pictures showed paracrystalline arrays and raft-like structures made up from double rows of rhodopsin molecules, with individual dimers breaking off at the end of the structures, and occasional monomers. The model of oligomerized rhodopsin derived from these studies and a previously reported structure of bovine rhodopsin (Palczewski *et al.* 2000) is shown in Fig. 3. The density of the receptors in tightly packed regions was between 35 000 and 50 000 monomers/ μm^2 , somewhat higher than that estimated previously (Chabre *et al.* 2003). However, based on earlier biophysical measurements and the concern over possible phase separation between proteins and lipids during sample preparation, it has been suggested that the observed structure does not correspond to the native conformation of nonactivated rhodopsin in dark-adapted rods, but is instead an artifact of the preparation process (Chabre *et al.* 2003, Chabre & le Maire 2005). Beyond this controversy regarding the *in vivo* relevance of the rhodopsin structure observed by AFM (Chabre *et al.* 2003, Fotiadis *et al.* 2003a,b), it should be kept in mind that rhodopsin molecules constitute more than 90% of all disk membrane proteins, which cannot be said of GPCRs in general, and consequently this structure may only be the hallmark of this specialized compartment. The above studies were repeated using ROS membranes isolated from mice heterozygous for the rhodopsin gene deletion, and the observed structure and receptor density showed no major changes compared with the wild-type mice, while the

Figure 3 Model of rhodopsin oligomers deposited at the Protein Data Bank (code 1N3M) based on data obtained from atomic force microscopy (Liang *et al.* 2003) and the crystal structure of bovine rhodopsin (Palczewski *et al.* 2000). In the insert, dashed lines indicate the rhodopsin dimer, which is enlarged in the figure. In the dimer, interdimeric contacts occur at helices 4 and 5.

volume of the ROS was smaller (Liang *et al.* 2004). In an artificial system, rhodopsin was able to self-associate into dimers even at a low receptor density, as assessed by FRET measurements, which argues for the dimerization of rhodopsin at lower surface densities (Mansoor *et al.* 2006).

The high-resolution crystal structures of rhodopsin do not show a consistent dimerization of the rhodopsin molecule. The first high-resolution crystallographic structure showed rhodopsin dimers in biologically irrelevant antiparallel organization (Palczewski *et al.* 2000), and other studies reported similar arrangements of rhodopsin crystals (Li *et al.* 2004). Although a recent study using new crystal forms of rhodopsin suggested an oligomerization contact site, the surface of this site is too small to predict stable interactions and different from the site predicted by a model based on the AFM studies, and it was suggested that it may mediate the secondary contacts between the rows of rhodopsin molecules in the AFM images (Lodowski *et al.* 2007). Very recently, the crystal structure of the human β_2 AR became available. Rasmussen *et al.* (2007) reported the structure of the receptor, which was crystallized in a lipid environment, bound to an inverse agonist in complex with a monoclonal antibody Fab fragment that binds to the third intracellular loop. In this crystal, the β_2 AR was found to be monomeric, in contrast to the reported rhodopsin crystal structures. In another study, the crystallization of the receptor was facilitated by inserting T4 lysozyme in place of the third intracellular loop (Cherezov

et al. 2007). This structure showed a multilayered arrangement of the receptors, and within each layer, the protein molecules formed arrays of parallel, symmetry-related dimers. The main contact point was between the charged amine group of Lys⁶⁰ in TM1 and the carboxylate of Glu³³⁸ in helix 8, a cytoplasmic helix formed in the membrane-proximal part of the tail, roughly perpendicular to the TM bundle, which is thought to be a common feature in all rhodopsin-like GPCRs (Katragadda *et al.* 2004). Similar associations between receptor molecules have been found in rhodopsin crystal structures as well (Schertler 2005, Salom *et al.* 2006). However, the dimerization interface of GPCRs is controversial, since other studies suggested different localizations, such as TM6 as the dimer interface for the β_2 AR (Hebert *et al.* 1996), TM4 was reported to mediate homodimerization of the D2 dopamine and C5a complement receptors, and TM1 and 2 were implicated in mediating the dimerization of the yeast α -factor receptor (reviewed in Kroege *et al.* (2003) and Breitwieser (2004)).

Receptor G-protein stoichiometry

All GPCRs, including rhodopsin, were initially assumed to exist and function as monomers, activating a heterotrimeric G-protein with a 1:1 stoichiometry. Most of the previously available biophysical and biochemical data supported this idea (reviewed in Chabre & le Maire (2005)). When the AFM

studies showed dimeric structures in the ROS membranes, it has been suggested that the functional unit may be composed of a rhodopsin dimer coupling to one G-protein (Liang *et al.* 2003). This model was based on the crystal structure of monomeric rhodopsin (Palczewski *et al.* 2000) and stated that the cytoplasmic surface of one receptor was too small to interact with all subunits of transducin on its own, while the combined surface of a rhodopsin dimer would provide a good docking site, one protomer interacting with the α - and the other with the $\beta\gamma$ -subunits (Filipek *et al.* 2004). A biochemical analysis of rhodopsin molecules showed that although both activated monomers and dimers were capable of activating transducin, the activation process was faster in the case of organized dimers (Jastrzebska *et al.* 2006), further supporting this model (Fotiadis *et al.* 2006). In the case of the B4 leukotriene type 1 (BLT1) receptor, a similar pentameric assembly was proposed, composed of a BLT1 dimer and the heterotrimeric G-protein (Baneres & Parelo 2003).

However, this concept has been challenged based on arguments that the crystal structures used for the construction of the above model are not sufficiently complete, especially regarding the interaction site between the receptor and the G-protein, and a model was proposed, in which a monomeric GPCR acts as the functional unit (Chabre & le Maire 2005). Recent data seem to be supporting the latter hypothesis, including a computational analysis, which states that monomeric dark rhodopsin holds the molecular determinants for transducin recognition (Dell'Orco *et al.* 2007), and a biochemical study in which monomeric rhodopsin was sufficient for fully functional transducin activation (Ernst *et al.* 2007).

Using very similar approaches, data from two workgroups further confirm the validity of a 1:1 stoichiometry between the GPCR and its G-protein. Transducin activation was studied in nanoscale lipid bilayers (Nanodisc), containing either one or two rhodopsin molecules and one transducin (Bayburt *et al.* 2007). It was shown that in this system a rhodopsin monomer is fully capable of highly efficient coupling to transducin, and that in Nanodiscs containing two receptors, only one was able to form a stable metarhodopsin II-transducin complex. In another paper, a monomeric β_2 AR was incorporated into a reconstituted high-density lipoprotein phospholipid bilayer particle together with the stimulatory heterotrimeric G-protein, G_s . The monomeric β_2 AR efficiently activates G_s in this system and displays GTP-sensitive allosteric ligand-binding properties, suggesting that a monomeric receptor in a lipid bilayer is the minimal functional unit necessary for signaling, and that the cooperativity of agonist binding is due to G-protein association with a receptor monomer and not receptor oligomerization (Whorton *et al.* 2007).

It has also been suggested that the arrestin molecule has the appropriate size and shape to interact with a rhodopsin dimer, and consequently this interaction would also have a 2:1 stoichiometry (Liang *et al.* 2003, Fotiadis *et al.* 2006). However, recent *in vivo* and *in vitro* experiments also argued

against this hypothesis, supporting the 1:1 stoichiometry between the receptor and arrestin (Hanson *et al.* 2007).

Functional studies

Perhaps the most important pieces of evidence in support of the concept of receptor dimerization and/or oligomerization came from functional studies. Several studies have reported co-internalization and modulation of the signaling activity of hetero-oligomerized GPCRs (for citations see Terrillon & Bouvier (2004), Prinster *et al.* (2005)). However, demonstration of the co-internalization of GPCRs has a limited value as an evidence for oligomerization. The internalization of receptors requires the association of the receptor with larger molecular complexes, such as the clathrin coat or caveolae, and association of non-oligomerized 'bystander' receptors with components of these complexes, leading to its co-internalization with another activated receptor, is difficult to rule out. Similarly, activation of the signal transduction of a receptor with another GPCR can occur with a number of different mechanisms, caused by the signaling of the activated receptor, rather than direct interactions due to oligomerization. However, studies on the trafficking of newly synthesized receptors, and modulation of the ligand binding by co-expressed receptors provided interesting findings in favor of the concept of receptor oligomerization.

Studies with family C GPCRs

The functional evidence, which gave a wide recognition to the concept of GPCR dimerization was the discovery that the function of metabotropic GABA_B receptors absolutely requires heterodimerization of GABA_{B1} and GABA_{B2} receptors (Jones *et al.* 1998, Kaupmann *et al.* 1998, White *et al.* 1998). It has been demonstrated that GABA_{B1} receptors are unable to reach the cell surface in the absence GABA_{B2} receptors, because GABA_{B1} receptors contain an endoplasmic reticulum retention signal, which is masked by heterodimerization with GABA_{B2} receptors (Couve *et al.* 1998). It is now widely accepted that GABA_{B1} and GABA_{B2} receptors work together as a functional unit in which agonists interact with the GABA_{B1} subunit, whereas the GABA_{B2} subunit is responsible for G-protein activation and efficient trafficking of the heterodimer to the cell surface (Galvez *et al.* 2001). The *in vivo* relevance of the functional importance of this interaction is well accepted and supported by a number of findings, including data with GABA_{B1} and GABA_{B2} receptor knockout mice, which demonstrated that the deletion of either gene leads to similar phenotypes and almost complete elimination of all GABA_B-mediated responses (Gassmann *et al.* 2004, Pin *et al.* 2007). In some brain areas GABA_B function is retained after genetic elimination of the GABA_{B2} receptors, which may suggest that the role of GABA_{B2} receptors in functional complementation of

GABA_{B1} receptors is not exclusive, and other proteins can substitute for this function (Gassmann *et al.* 2004).

The essential role of heterodimerization in the function of other family C receptors has also been demonstrated. The functional units of umami and sweet taste receptors are also heterodimers of T1R1 and T1R3, T1R2 and T1R3 receptors respectively and similar to GABA_B receptors, each of these subunits serve specific functions in terms of ligand recognition and G-protein activation (Nelson *et al.* 2001, 2002, Xu *et al.* 2004). The physiological relevance of this heterodimerization was demonstrated in studies using knockout mice, which showed that elimination of the T1R1 and T1R2 gene affects sweet and umami taste recognition respectively, whereas genetic deletion of the T1R3 gene impairs both functions (Damak *et al.* 2003, Zhao *et al.* 2003).

Studies with family A and B GPCRs

Functional data on GPCR dimerization are not limited to family C receptors. Naturally occurring N-terminally truncated variant of the family B pufferfish calcitonin receptor can heterodimerize with the normal receptor protein and can attenuate the ligand-induced intracellular signaling without affecting its plasma membrane targeting and ligand-binding properties, possibly by changing the conformation of the cytoplasmic regions of the receptor that interacts with the G-proteins (Nag *et al.* 2007). The mechanism may be similar to the one found in the case of GPR50, an orphan GPCR, which has been shown to heterodimerize with, and specifically inhibit the MT1 melatonin receptor (Levoye *et al.* 2006). It has also been demonstrated that heterodimerization of family A α_{1D} -adrenoceptors with α_{1B} -adrenoceptors is necessary for proper cell surface expression of the α_{1D} -adrenoceptor (Hague *et al.* 2004b), and that co-expression and heterodimer formation of α_{1D} - and α_{1B} -adrenoceptors lead to an altered pharmacological profile of these receptors, which may explain that α_{1D} -adrenoceptor are pharmacologically undetectable in tissues, which express abundant quantities of its mRNA (Hague *et al.* 2006).

In most systems dimerization or oligomerization of GPCRs is agonist independent and occurs early during receptor biosynthesis, probably in the endoplasmic reticulum. In some cases, agonist effect on receptor oligomerization has been reported, but these changes may reflect the effects of agonist-induced conformational changes on the stabilization of preexisting oligomers or RET efficiency (Bulenger *et al.* 2005). However, as detailed above, compelling evidence suggests that heterodimer formation is required for many family C, and some family A receptors, to reach the cell surface. This might be a more general phenomenon since a large number of other GPCRs, such as adrenocorticotropin receptors, adenosine, bitter taste, and olfactory receptors, do not express well in heterologous expression systems (Noon *et al.* 2002, Clark *et al.* 2003, Minneman 2007). Although the tissue-specific mechanisms leading to the cell surface expression of

these receptors has not been fully elucidated, hetero-oligomerization with the β_2 -adrenergic receptor has been reported to facilitate the cell surface expression of olfactory receptors (Hague *et al.* 2004a). It is possible that, at least in some tissues, proper expression of these receptors requires the presence of other GPCRs, but the role of other chaperons or chaperon-like proteins cannot be excluded in this process.

Many GPCRs express well in a wide range of heterologous expression systems. Apparently, these receptors do not need hetero-oligomerization to avoid the quality control mechanisms of the endoplasmic reticulum. It has been proposed that oligomerization may be a common requirement for GPCRs to pass quality checkpoints along the biosynthetic pathway, with homo-oligomerization being the general rule, and hetero-oligomerization being a special case for some receptors (Bulenger *et al.* 2005). Although this hypothesis needs additional experimental validation and other exceptions may exist, it is an attractive hypothesis which can explain the need for hetero-oligomerization for cell surface expression of some receptors, whereas most receptors could reach the cell surface as homo-oligomers. In the latter case the role of homo-oligomerization in cell surface targeting may explain the dominant negative behavior of pathogenic GPCR mutations, which cause intracellular retention of the mutant receptor as well as the co-expressed wild-type receptors and decrease their expression and signaling (Zhu & Wess 1998). This can result in a dominant transmission of diseases in heterozygotes (Tao 2006), such as in the case of partial thyroid-stimulating hormone (TSH) resistance due to heterozygous inactivating mutations in TSH receptor gene (Calebiro *et al.* 2005). Such dominant-negative effect can be advantageous as well: a truncated form of the chemokine receptor type 5 (CCR5), CCR5 Δ 32 can oligomerize with the wild-type CCR5 in the ER, causing an intracellular retention of the oligomer (Benkirane *et al.* 1997). Since CCR5 is a major co-receptor for the human immunodeficiency virus (HIV), individuals carrying this mutation even in a heterozygous form are partially protected from the infection and display a slower progression of the disease. While the role of oligomerization in this effect is not entirely undisputed (Venkatesan *et al.* 2002), further studies indicated that in addition to CCR5, CCR5 Δ 32 can also oligomerize with and reduce the expression of chemokine receptor type 4 (CXCR4), which is also implicated in HIV entry into the cells (Agrawal *et al.* 2004).

Many GPCRs have alternative splice variants (often truncated forms of the wild-type receptor), and these can also have a regulatory effect on the full-length molecule; a splice variant of the dopamine D3 receptor, D3nf, truncated before the sixth TM, has been shown to oligomerize with the wild-type receptor, thereby reducing its expression (Karpa *et al.* 2000). The relative expression of D3 and D3nf was found to be different in high and low responders to novelty in an animal model, which may indicate the physiological relevance of this splice variant (Pritchard *et al.* 2006).

Studies on ligand binding

It has been known for a long time that the interaction of GPCRs with other molecules can lead to an allosteric modulation of the ligand binding of the receptor. The 'classical' examples of these modulations are interactions with G-proteins and Na^+ ions (Catt & Dufau 1977). However, if GPCRs exist as dimers or oligomers, ligand binding to one receptor may lead to conformational changes which affect the ligand binding of the partner receptor. In case of receptor homo-oligomers, such interactions would be detected as positive or negative cooperativity of the ligand binding. In fact, early studies demonstrated cooperativity of the ligand binding of various GPCRs, such as angiotensin (Sraer *et al.* 1977), β -adrenergic (Limbird & Lefkowitz 1976), opiate (Davis *et al.* 1977), TSH (Tate *et al.* 1975), and other (Baxter & Funder 1979) receptors. However, interpretation of these observations were ignored or considered simply the consequence of receptor–G-protein interaction, since GPCRs were widely regarded as monomeric entities at the time. Mesnier and Baneres have demonstrated an allosteric interaction between protomers of a wild-type and mutant BLT1 receptor upon leukotriene B4 binding using a spectroscopic approach (Mesnier & Baneres 2004).

Vassart *et al.* designed a number of experiments to study whether the presence of agonists affects the kinetics of ligand dissociation after an infinite dilution of a pre-bound labeled ligand. They were able to show in all three families of GPCRs, that in case of the receptors that form homo-oligomers, the presence of unlabeled ligand facilitated the dissociation rate of the tracer (El Asmar *et al.* 2005, Urizar *et al.* 2005, Springael *et al.* 2006, 2007). Under the experimental conditions used in these studies, rebinding of the tracer is unlikely and the observed effects can be attributed to allosteric interactions between the subunits of receptor dimers or oligomers. Interestingly, both agonists and antagonists could evoke faster ligand dissociation (i.e. negative cooperativity), indicating that full receptor activation is not required to cause allosteric interactions between receptor oligomers. Allosteric interactions have also been demonstrated between hetero-oligomerized chemokine receptors (Springael *et al.* 2006), and studies with GABA_B receptors also indicate that although the different subunits have specific functions, the other subunit can allosterically stimulate the hormone binding of the GABA_{B1} subunit or the G-protein activation by the GABA_{B2} subunit (Galvez *et al.* 2001). These interactions provide evidence for the functional relevance of GPCR dimerization or oligomerization, since these interactions can be demonstrated in native tissues (Springael *et al.* 2007).

A cross-inhibition of GPCRs also provides indirect evidence to support the concept of receptor oligomerization. Perhaps the most striking example of this effect is the cross-inhibition of β -adrenergic and AT_1 angiotensin receptors (Fig. 4), which was independent of allosteric modulation of the ligand binding of these receptors (Barki-Harrington *et al.*

Figure 4 A cross-inhibition of β -adrenergic (BAR) and AT_1 receptors (AT_1R ; Barki-Harrington *et al.* 2003). (A) Pharmacological inhibition of the BAR can inhibit the signaling of the heterodimerized AT_1R and (B) the inhibition of the AT_1R can inhibit the signaling of the activated BAR.

2003). Interestingly, whereas β -adrenergic receptors are sensitive to valsartan, an AT_1 receptor blocker, in the heart, they are unaffected by this drug in endothelial cells, which express a far greater ratio of β -adrenergic receptor to AT_1 receptors (Barki-Harrington *et al.* 2003). Using mutant receptors that were insensitive to the AT_1 receptor blocker, candesartan (Szidonya *et al.* 2007), or a G-protein coupling-deficient receptor (Gaborik *et al.* 2003, Wei *et al.* 2003), it was also demonstrated that cross-inhibition of AT_1 receptor homo-oligomers can also occur (Karip *et al.* 2007). The inhibitory effect observed in these studies may reflect the notion that the optimal binding of a G-protein requires two interacting GPCRs (Filipek *et al.* 2004), but it is also possible that the conformational change induced by the binding of the inverse agonists used in these studies causes allosteric inhibition of the associated receptor. In fact, a cross-inhibition of the above-mentioned mutant AT_1 receptors (Karip *et al.* 2007) suggest that an activated AT_1 receptor associated with a

G-protein coupling-deficient receptor can stimulate inositol phosphate signaling.

These and other functional data clearly indicate that a functionally important molecular interaction between GPCRs does occur. However, it will probably remain a matter of debate for more time, whether these interactions have a well-defined structural base, such as formation of receptor oligomers, or these interactions are indirect, and involve other proteins (or lipids). Despite this controversy, in our opinion receptor oligomerization is a useful paradigm to explain pharmacological properties of receptors that require direct or indirect interactions between GPCR molecules. Understanding of the functionally important interactions between GPCRs and their role in tissue-specific pharmacological actions of GPCR ligands is an important task. Recently, IUPHAR has worked out the criteria to define functionally relevant hetero-oligomeric interactions between GPCRs (Pin *et al.* 2007). According to these recommendations in order to define hetero-oligomeric interactions between GPCRs at least two of the following three conditions should be met. They are: 1) evidence for physical association in native tissue or primary cells (based on immunological colocalization or co-immunoprecipitation), 2) identification of a specific functional property for the hetero-oligomeric receptors in native tissue and 3) the use of knockout animals or RNAi technology to provide information on the existence of hetero-oligomeric GPCRs *in vivo* (Pin *et al.* 2007). The consistent application of these criteria will help to clarify the physiological and pharmacological relevance of the interactions between GPCRs.

Concluding remarks

In summary, evidence obtained with a wide range of different methods is accumulating, which suggest that GPCRs function as dimers or oligomers. Although individual evidences can be, and most of them were, challenged based on methodical grounds, the multitude of available positive data suggest that some kind of dimerization, oligomerization, or clustering of most GPCRs does occur. However, it is apparently very difficult to obtain decisive evidence, whether these receptors are organized into well-defined structures, such as dimers or oligomers, and interact directly; or function in larger molecular complexes, where propagation of the information occurs via complex molecular networks. It is likely that this issue will not be fully resolved until the necessary tools are developed to obtain high-resolution snapshots of GPCRs in their native environment. Although it may take a lot more time for structural biologists to achieve this goal, dimerization, or oligomerization of GPCRs is a very useful paradigm for pharmacologists to study properties of receptors, which require functionally important clustering, such as allosteric modulation of ligand binding, co-internalization, altered signaling properties, or

cross-inhibition. An elucidation of these interactions is an important immediate task since these are critical to understand the pharmacological effects of drugs targeted to receptors and to elucidate the physiological mechanism of action of hormones and other mediators that target GPCRs.

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